Morphological and Genetic Divergence in Three Populations of *Anthocoris antevolens* (Hemiptera: Heteroptera: Anthocoridae)

DAVID R. HORTON,[1] THOMAS R. UNRUH, TAMERA M. LEWIS, AND KELLY THOMSEN-ARCHER

USDA–ARS, 5230 Konnowac Pass Road, Wapato, WA 98951

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ABSTRACT *Anthocoris antevolens* White (Hemiptera: Heteroptera: Anthocoridae) is a widespread predatory bug in North America that exhibits substantial geographic variation in coloration, body measurements, size and shape of the male genitalia, pubescence, and sexual behavior. Earlier behavioral studies with three populations (including two populations that are sympatric in central Washington) showed that there was limited or no successful mating between insects from nonlike populations, despite vigorous mating attempts by males. The current study shows that males from those three populations diverge also in size and shape of claspers, length of the phallus, body measurements, and pubescence. Divergence extends to the two sympatric populations. Analysis of mitochondrial DNA shows that phenotypic divergence is associated with genetic divergence. Results reported here, in combination with the earlier published mating trials, support statements made elsewhere by us that *A. antevolens* is actually a complex of an unknown number of externally similar species.

KEY WORDS morphometrics, genitalia, cryptic species, geographic variation, mtDNA

The predatory bug *Anthocoris antevolens* White (Hemiptera: Heteroptera: Anthocoridae) is a widespread species found throughout Canada and Alaska, south into the New England states and northern plains states east of the Rockies, and south into Arizona and southern California west of the Rockies (Kelton 1978, Henry 1988). Of the 12 species of *Anthocoris* listed for North America (Henry 1988), *A. antevolens* is geographically the most widespread. The bug associates often with deciduous trees and shrubs, especially in the Salicaceae and Rosaceae (Horton et al. 2004), and it can be an important source of biological control in orchard systems of the Pacific northwest (McMullen and Jong 1967, Horton and Lewis 2000).

A. antevolens shows substantial geographic variation in size, coloration, pubescence, sexual behavior, size or shape of the male's clasper, and length of the male's phallus (Horton et al. 2005, Horton and Lewis 2005, unpublished data). The variation is now known to encompass even sympatric populations, and population divergence has been shown to be associated with reproductive incompatibility between those sympatric populations (Horton et al. 2005, Horton and Lewis 2005). Thus, it has become clear that *A. antevolens* is actually a complex of an unknown number of reproductively isolated species whose external characteristics nonetheless key them to *A. antevolens* in available keys (Hill 1957, Kelton 1978).

Horton and Lewis (2005) showed that *A. antevolens* collected in western Yakima, WA, from Oregon white oak, *Quercus garrayana* Douglas (Fagaceae), was reproductively isolated from *A. antevolens* collected from neighboring stands of willow, *Salix* spp. (Salicaceae). Males from the two populations differed in shape of the clasper and length of the phallus. Morphological differences and reproductive incompatibility were maintained in the laboratory in consecutive generations reared on a common diet (Horton and Lewis 2005). Further examination of bugs from the *Salix* source suggested that this population may itself be composed of two distinct types, based upon a limited examination of male genitalia and pubescence on the hemelytra (unpublished data). Behavioral studies were then done that confirmed that two reproductively incompatible populations do indeed occupy *Salix* (and other host plants such as pear, *Pyrus communis* L. [Rosaceae]) throughout the Yakima Valley (Horton et al. 2005).

Here, we explore whether the two Yakima populations co-occurring on both *Salix* and *Pyrus* spp., and shown earlier to be reproductively incompatible (Horton et al. 2005), also demonstrate quantifiable differences in body measurements, size and shape of the male genitalia, pubescence, and molecular genetic composition. As in the Horton et al. (2005) study, we have included for comparison a third population collected from *Salix* ≈120 km west of the two Yakima populations. Females from that third population in laboratory trials were shown to be successfully inseminated by males from one of the two Yakima types, albeit at low rates, but they were found to be completely incompatible with males from the second

[1] Corresponding author, e-mail: horton@yarl.ars.usda.gov.

Yakima population (Horton et al. 2005). Specific objectives are to determine whether the reproductive incompatibilities noted in Horton et al. (2005) are reflected in genetic and morphological traits within the three populations.

Materials and Methods

Source of Insects and Rearing. Insects used in the current study were male siblings of those used in the mating studies described previously (Horton et al. 2005), with some additional material collected in a subsequent year (see below). In all cases, bugs were the first-generation male offspring of field-collected females. Insects from three populations (described in Horton et al. 2005) were examined. The Alder Lake (AL) population was collected in June 2002 from *Salix* growing along a 20-km stretch of Highway 7 between Mineral Lake and Alder Lake, WA (46° 71′ N, 122° 22′ W). The site is west of the Cascade Crest. A second population (GC) was collected in June 2002 from ≈20 pear trees growing at the Apple Tree Golf Course in western Yakima, WA (46° 57′ N, 120° 61′ W). The site is ≈120 km east of the AL site and occurs east of the Cascade Crest. The third population (UG) was collected June 2002 from a 100-m stretch of *Salix* growing along the Yakima River just southeast of Union Gap, WA (46° 52′ N, 120° 47′ W). The UG and GC sites are separated by 12 km. However, we occasionally find UG and GC type bugs co-occurring throughout the Yakima Valley on *Salix* spp. and *Pyrus communis* (unpublished data). We have examined enough insects from both the UG and GC populations that we can now differentiate between the two phenotypes based upon external characteristics (the GC bugs are generally darker and have less noticeable pubescence); we carefully examined our field-collected material before beginning the studies described below, to ensure that parental bugs were not a mix of the two phenotypes within either the GC or UG collections.

For each population, 10 mated females were placed individually in small cages (135-ml ventilated vials) containing prey [nymphs and eggs of pear psylla, *Cacopsylla pyricola* (Förster) (Homoptera: Psyllidae)], and small pear seedlings. The bugs readily oviposited into the pear seedlings. Offspring were reared at 22–24°C and a photoperiod of 16:8 (L:D) h. Large nymphs were removed from the cages and placed individually in petri dishes (9 cm in diameter) lined with filter paper and containing psylla-infested pear leaves. As the nymphs eclosed to the adult stage, date of maturation was noted, and the insects were sexed. We randomly chose one son per female for examination. Males were 3–5 d in age when they were killed (by freezing) and mounted on points. In summer 2004, an additional five females per each population were collected from the field to provide an additional five (nonsibling) males per population, by using rearing methods identical to those described above. These males, and the original 10 males per population from the 2002 collections, were then used in the analyses summarized below, producing sample sizes of 15 males per population.

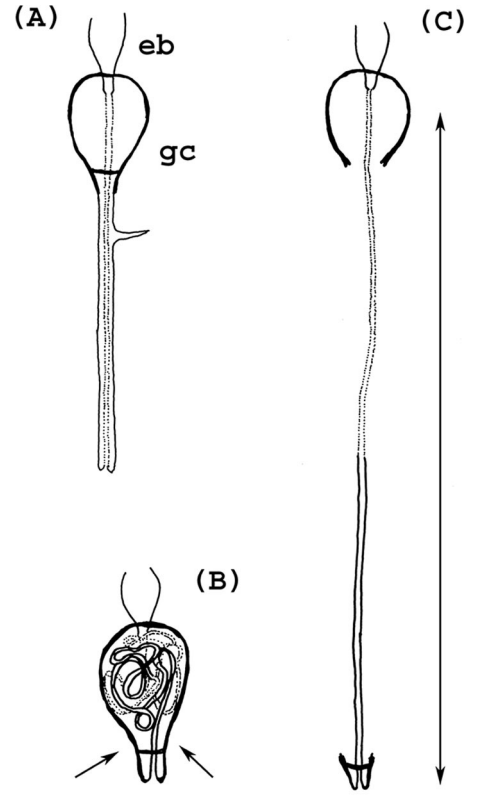

Fig. 1. (A) Fully inflated phallus. (B) Stored phallus within the genital capsule, with arrows showing points of dissection. (C) Phallus pulled from genital capsule for measuring (also see Horton and Lewis 2005). eb, ejaculatory bulb; gc, genital capsule.

Male Genitalia. We estimated length of the phallus and described size and shape of the left clasper for each specimen (Horton and Lewis 2005). The phallus in *A. antevolens* is a thin, membranous two-walled organ (Fig. 1) that must be fully inflated within the female's copulatory tube to allow insemination (Horton and Lewis 2005). Phallus length was estimated by dissecting the uninflated organ from the genital capsule and measuring length of the dissected organ beneath a dissecting microscope. Measurement has high repeatability (Horton and Lewis 2005). A single individual did all dissections and measurements. The dissected organ is measured as a single-walled structure (the form in which the phallus is stored in the genital capsule), rather than the two-walled structure of the inflated organ (Fig. 1). Thus, each phallus length reported below (and in Horton and Lewis 2005) is approximately twice the length of what the fully inflated organ would actually measure.

Clasper size and shape also were described for each specimen. The clasper is a sclerotized organ at the end of the male's abdomen, which the male partially in-

serts into the female's copulatory tube during mating. Once the clasper has been inserted, the male then inflates his phallus, which is channeled initially into the female along a groove on the clasper (Carayon 1972). Clasper shape is used extensively to confirm species' identifications within the Anthocoridae, including within *Anthocoris* (Hill 1957, Kelton 1978). Specimens of *Anthocoris* in this study all had the typical *A. antevolens* clasper (Kelton 1978) but with measurable differences in size and shape among the three populations (see below).

The clasper for each male was removed using microdissection tools and placed on a microscope slide (full details of methods in Horton and Lewis 2005). Each slide was placed beneath a Leica DMLS compound microscope (Leica, Bannockburn, IL) and photographed with a Spot Insight color digital camera (Diagnostic Instruments, Sterling Heights, MI). The photograph was transferred onto a computer, and the image was digitized using a program available through the morphometrics web page of the State University of New York (Rohlf 2001, http://life.bio.sunysb.edu/morph). We began each digitization at the tip of the hook and digitized in the same direction for each clasper (a digitized clasper is shown in Horton and Lewis 2005). One person did all of the digitizations. Each digitization resulted in 70–90 points arranged approximately evenly around the perimeter of the clasper.

Statistical Analyses. Phallus length was compared among populations using analysis of variance (ANOVA). The analyses were done in PROC GLM (SAS Institute 2001). Contrasts were then obtained between all possible pairs of populations (GC versus UG, GC versus AL, and UG versus AL), by using the PDIFF command in PROC GLM (SAS Institute 2001); contrasts were judged to be significant if P values were <0.017 (i.e., 0.05 divided by the number of contrasts). Clasper shape was described quantitatively using elliptic Fourier analysis, which is useful for describing the shape of objects not easily measured using landmark-based methods (Liu et al. 1996, McLellan and Endler 1998, Arnqvist and Danielsson 1999). The method has been used to describe outlines of genitalia in insects (Liu et al. 1996, Monti et al. 2001), including in *A. antevolens* (Horton and Lewis 2005). We used the program in Rohlf and Ferson (1992) to compute Fourier coefficients from the X-Y coordinates obtained for each digitized outline. Analyses were made invariant to clasper orientation, rotation, and choice of starting point, using options available in the Rohlf and Ferson (1992) package. Twenty harmonics from the Fourier analysis successfully described clasper shape (Horton and Lewis 2005). The 80 coefficients from these 20 harmonics were then used in a principal components analysis (PCA), to distill the coefficients into a smaller set of descriptors (Ferson et al. 1985, Horton and Lewis 2005). The PCA was done using the correlation matrix. We used the PROC PRINCOMP program in SAS (SAS Institute 2001) for the PCA. We then used ANOVA on each of the first three principal components to test whether clasper size or shape differed among populations (Horton and Lewis 2005).

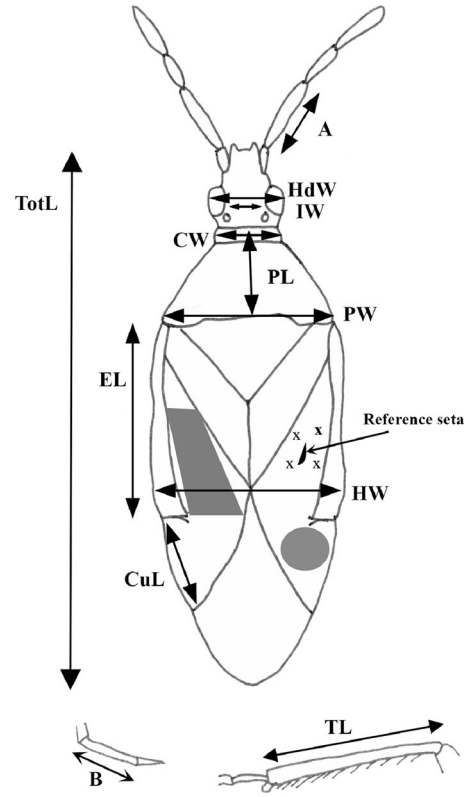

Fig. 2. Twelve mensural characters (arrows) measured in male *A. antevolens* from three populations. Gray trapezoid and circle show the locations on hemelytra in which setal densities were estimated (all setal characteristics actually recorded for right forewing; trapezoid is shown on left forewing to reduce figure clutter). Setal length determined for the four setae (shown as x's) located in proximity to the long reference seta found approximately at mid-point of endocorium (see text). TotL, total length; HdW, head width; IW, interocular width; CW, collar width; PW, pronotum width; PL, pronotum length; HW, hemelytra width; CuL, cuneus length; A, length of second antennal segment; B, length of second beak segment; TL, tibial length.

Body Measurements. Twelve body measurements were obtained for each specimen (Fig. 2). The measurements were chosen in part because preliminary examination of specimens obtained from a number of geographic locations suggested that these characteristics may vary among locations. Some measures (e.g., interocular width) were chosen because they commonly are used in morphometric or systematic studies within Heteroptera (e.g., Schwartz and Foottit 1998). Measurements were taken using a dissecting microscope equipped with an ocular micrometer. The measurements were made on the mounted specimens.

Tibial length (TL in Fig. 2) was measured on the rear left leg. Pronotum length (PL) included the collar, because the demarcation between the pronotum and collar was not always easily defined. Hemelytra

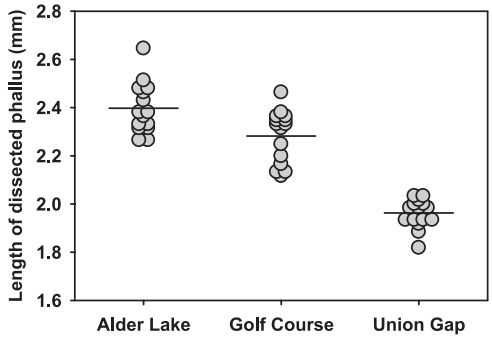

Fig. 3. Lengths of dissected phallus and associated means for males from three populations of *A. antevolens.*

width (HW) was measured at the point on the body of maximum width. Interocular width (IW) was measured at the point of minimum distance between the eyes. Length of the beak (B) was recorded for the penultimate segment. The antennal measurement (A) was recorded for the second segment.

Statistical Analysis. Analysis of covariance (ANCOVA) was used to test whether body measurements differed among populations. Overall length (TotL in Fig. 2) was used as the covariate. Analyses were done using PROC GLM in SAS (SAS Institute 2001). Due to the number of statistical tests made (one analysis for each measure in Fig. 2), significance of population effects for a given analysis was determined using a sequential Bonferroni adjustment (Rice 1989). This approach is suitable for assessing significance of individual tests in a table containing a number of independent or non-independent statistical tests. The adjustment reduces probability of obtaining false significance in tests, but does so without a large sacrifice in statistical power. After a significant ANOVA, population means were separated using the PDIFF command, again with significance set at $P = 0.017$.

Pubescence. Length and density of setae on the hemelytra are used in keys to separate *A. antevolens* from a closely related species, *Anthocoris musculus* (Say) (Hill 1957, Kelton 1978). We have found these characters to be highly variable among populations of *A. antevolens.* We measured length and density of setae on a section of the endocorium (Fig. 2, gray trapezoid), and density of setae on a section of the cuneus (Fig. 2, gray circle) in each of the 15 specimens per population. For each bug, the right forewing was removed and mounted dorsal side up in 75% ethanol on a microscope slide. The slide was placed beneath the compound microscope and photographed.

Setal Density: Endocorium. The forewing was photographed at 100× magnification, and the photograph was printed on a color printer. A line was drawn on the photograph through the cuneal fracture (=costal fracture) to intersect with the wing membrane; a second line 90 mm anterior of the first line (and parallel to the first line) was then drawn on the photograph. The median furrow and anal furrow (claval suture) were traced. The four lines produced a parallel-sided quadrilateral (gray trapezoid in Fig. 2; note that the trapezoid is actually illustrated for the left wing, just to reduce clutter in the figure). Setal bases were then counted for the area defined by the trapezoid. Area of the quadrilateral was estimated for each specimen by measuring the length of the four sides and calculating area using the formula for area of a trapezoid. Setal density was expressed as numbers of setal bases per unit area.

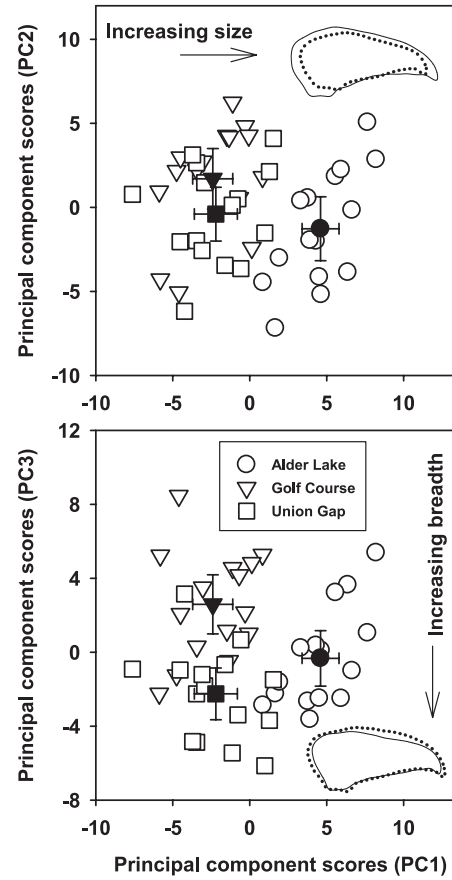

Fig. 4. Scatter plots relating scores from first and second principal components (top) and from first and third principal components (bottom). Filled symbols depict population mean scores and 95% confidence intervals. Reconstructed outlines of the claspers are shown for opposite ends along PC1 (top), to illustrate differences in clasper size; and for opposite ends along PC3 (bottom), to illustrate differences in clasper breadth or robustness.

Setal Density: Cuneus. The forewing was photographed at 200× magnification. The photographs were printed on a color printer. A plastic petri dish bottom (9 cm in diameter) was placed at the approximate center of the cuneus, ≈25 mm from the cuneal break in the photograph. We counted the number of setal bases within this circle. Density of setae was expressed as number of setal bases per unit area.

Setal Length: Endocorium. The forewing was photographed at 200× magnification. In *A. antevolens,* there is generally a distinctive long seta located about

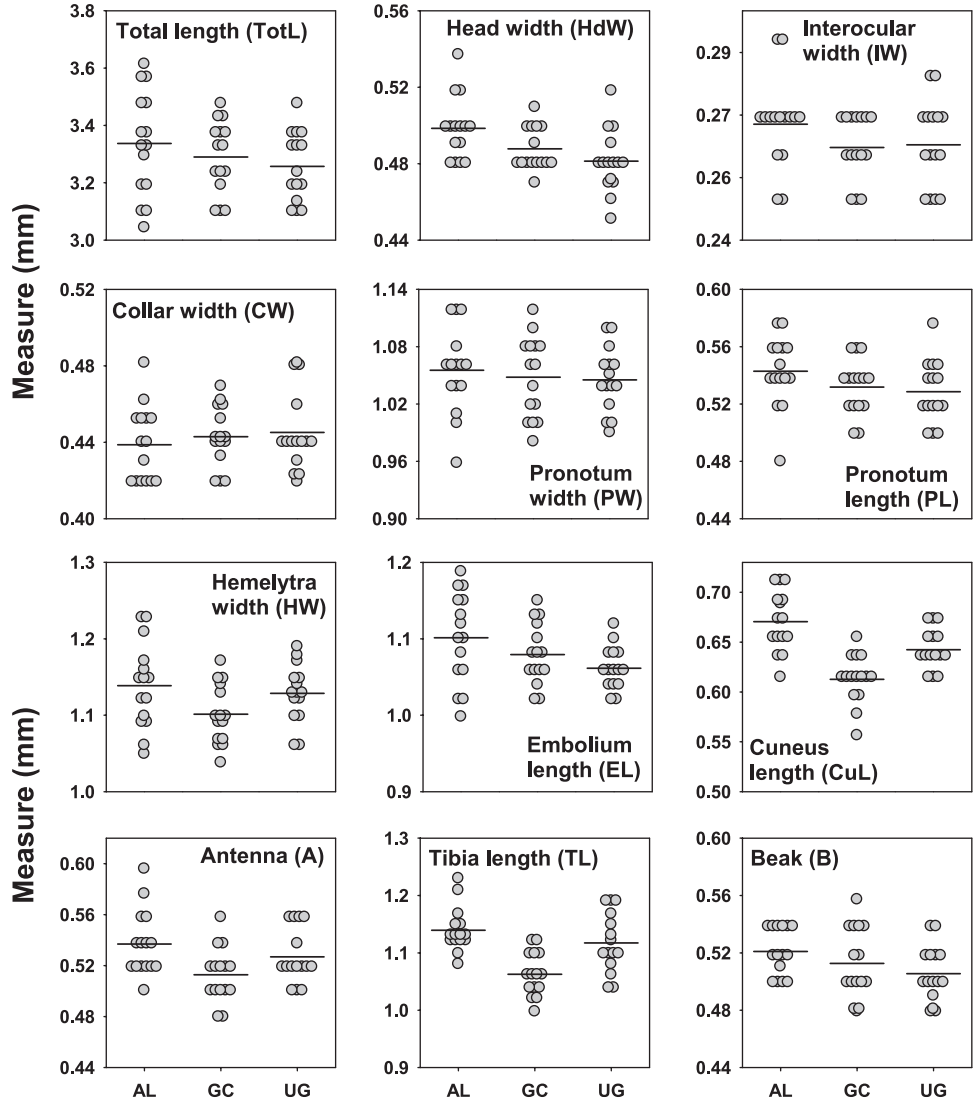

Fig. 5. Measurements for 12 mensural characters and associated means (horizontal lines) for males from three populations of *A. antevolens* (see Source of Insects and Rearing for description of population abbreviations).

halfway between the cuneal fracture and the point at which the epipleural fold crosses the median furrow, midway between the exocorium (embolium) and clavus. We used this seta as a reference to locate the portion of the wing within which setal lengths were to be determined (Fig. 2, Reference seta). We chose the four setae nearest the reference seta to measure (the x's near the reference seta in Fig. 2) and averaged those four lengths to obtain a single measure of setal length for each specimen.

Statistical Analyses. Mean density or length of setae was compared among populations using ANOVA. Population separation after a significant ANOVA was made using the PDIFF command. Analyses were done using PROC GLM (SAS Institute 2001).

Mitochondrial DNA (mtDNA). The same specimens used in the morphometric analyses were used for mtDNA sequencing. Thus, the 15 specimens within a population each came from a different field-collected mother. *Anthocoris nemoralis* (F.), an Old World species that has colonized western North America, was used as an outgroup; the specimen was collected from western Washington in May 2002. Three legs from one side of each pinned specimen were used in the DNA extractions. The extractions were done using the DNeasy kit (QIAGEN, Valencia CA) and manufacturer's instructions. DNA sequences were collected from the two mitochondrial genes, cytochrome oxidase subunit 1 (CO1) and cytochrome B (CytB), by using standard methods. The primers C1-J-1751 [GGATCACCTGATATAG CATTCCC] with C1-N-2191 [CCCGGTAAAATTA AAATATAAACTTC] were used to amplify ≈450 bp of the CO1 gene; CB-J-10933 [TATGTACTACCATGAG GACAAATATC] with CB-N-11367 [ATTACACCTC

CTAATTTATTAGGAAT] were used to amplify ≈450 bp of CytB. Primer names and numbering correspond to those reported in Simon et al. (1994). Amplifications were performed in 25-μl reaction volumes containing 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 3.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs (Boehringer Mannheim, Ridgefield, CT), and 0.625 U of *Taq*DNA polymerase, with 0.2 μM of each primer and 2 μl of template DNA. The polymerase chain reaction (PCR) temperature program followed specific protocols: 95°C for 120 s and 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 45 s. PCR products were used as sequencing templates with the same primers.

Double-stranded PCR products were purified with the QIAquick PCR purification kit (QIAGEN) or with Exo-Sap-it (USB Corp., Cleveland, OH). Cycle sequencing was done using ABI Big Dye chemistry (ABI PRISM BigDye Terminator version 3.1 cycle sequencing kit, Applied Biosystems, Foster City, CA). One-eighth reactions were used to produce 12 μl of cycle sequencing product, using 1 μl of the ABI reaction mix. Postsequencing cleanup was done with Centrisep columns (Princeton Separations Inc., Adelphia, NJ). Products were separated and visualized using an ABI PRISM 310 Genetic analyzer and associated software. All fragments were sequenced in both directions, and discrepancies between directions were resolved using Chromas-2 (Technelysium Pty Ltd., Tewantin, Queensland, Australia) to examine peaks generated by the base calling software.

Sequence alignments were created by eye in the program GeneDoc (http://www.psc.edu/biomed/genedoc). Sequences were trimmed to 425 bp for CO1 and 341 bp for CytB, retaining regions that showed consistent sequence and could be unambiguously aligned. CytB was trimmed significantly more than CO1 because of difficulties in obtaining good full-length sequence, especially in the AL and GC populations.

Gene trees reflecting both genetic similarity of the mitochondrial sequences and preliminary phylogenetic hypotheses were created using several algorithms, including neighbor-joining (NJ) implemented in MEGA3 (Kumar et al. 2004), NJ and maximum parsimony in PAUP (Swofford 2003), maximum likelihood in Phylip (DNAML; Felsenstein 1989, 2005), and Markov chains (MRBAYES; Huelsenbeck and Ronquist 2001). Unrooted trees were evaluated for the two mtDNA sequences both separately and combined.

Voucher Specimens. Voucher specimens for each population have been deposited with the M.T. James collection (Washington State University, Pullman, WA).

Results

Male Genitalia. Length of the dissected phallus differed among populations (Fig. 3; $F_{2, 42} = 86.9$; $P < 0.0001$). The phallus was shorter in males from the UG population than in males from the other two populations ($P < 0.0001$ for both comparisons). Differences were significant also between males from the AL and GC populations ($P = 0.002$).

PCA of Fourier coefficients from the digitization of claspers led to separation of populations along the first principal component (Fig. 4; $F_{2, 42} = 44.6$; $P < 0.0001$) and the third principal component (Fig. 4; $F_{2, 42} = 12.1$; $P < 0.0001$); population effects were only weakly significant along the second principal component ($P = 0.044$), due to the substantial overlap of populations along this axis (Fig. 4, top). The first principal component seems to express overall size effects and indicates that males from the AL population had larger claspers than males from the other two populations (Fig. 4; $P < 0.0001$ for both contrasts). There was no difference in clasper size between males from the GC and UG populations ($P = 0.83$). The third principal component (Fig. 4, bottom) seems to express variation in breadth or robustness of the clasper and indicates that males from the UG population had more robust or broader claspers than males from the other populations ($P < 0.005$ for both comparisons). Outlines of claspers reconstructed from the Fourier coefficients (Rohlf and Ferson 1992) are shown to illustrate results at opposite ends along the first principal component (top) and at opposite ends along the third principal component (bottom), illustrating divergence in clasper size and robustness, respectively.

Table 1. *F* and *P* statistics from ANOVA (total length) and ANCOVA (all other body measures, by using total length as a covariate) testing for population effects

Measure	*F*	*P*
Total length (TotL)	1.1	0.35
Head width (HdW)	3.8	0.03
Interocular width (IW)	1.0	0.39
Collar width (CW)	0.6	0.54
Pronotum width (PW)	0.04	0.96
Pronotum length (PL)	1.2	0.31
Hemelytra width (HW)	3.6	0.035
Embolium length (EL)	2.0	0.15
Cuneus length (CuL)	21.3	<0.0001*
Antenna (A)	4.3	0.02
Tibia length (TL)	15.6	<0.0001*
Beak (B)	1.0	0.37

Body measures defined in Fig. 2. df = 2, 42 for ANOVA and df = 2, 41 for ANCOVAs.

* Asterisk indicates significance following sequential Bonferroni adjustment.

Body Measurements. Of the 12 body measurements (Fig. 2), both cuneus length and tibial length differed significantly among populations by ANCOVA followed by sequential Bonferroni adjustment (Fig. 5; ANCOVA results in Table 1). All three population comparisons were significant for cuneus length ($P < 0.014$ for all three comparsions). Cuneus lengths for males from the GC population were noticeably smaller than lengths in males from the other two populations (Fig. 5). Males from the GC population also had shorter tibias than males from the other two populations ($P < 0.0001$ for both comparisons); tibia lengths were similar in AL and UG males ($P = 0.47$).

Pubescence. Length of setae on the endocorium differed among populations (Fig. 6; $F_{2, 42} = 195.6$; $P <$

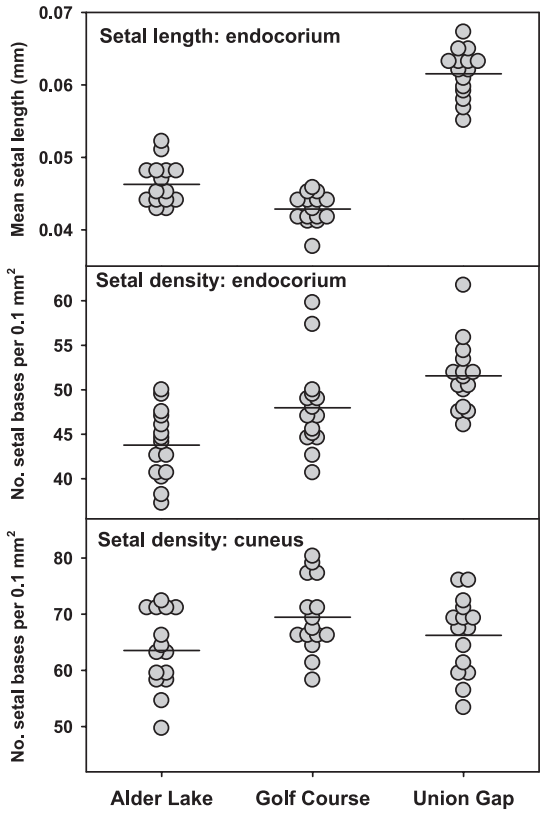

Fig. 6. Setal lengths and densities on endocorium, and setal densities on cuneus, with associated means (horizontal lines) for males from three populations of *A. antevolens*.

0.0001). Setae were noticeably longer in males from the UG population than in males from the other two populations ($P < 0.0001$ for both contrasts). Setae were shorter in males from the GC population than males from the AL population ($P = 0.002$). Density of setae on the endocorium also differed among populations (Fig. 6; $F_{2, 42} = 12.7$; $P < 0.0001$). Density was highest in males from the UG population and lowest in males from the AL population (three contrasts, each significant at $P < 0.016$). Density of setae on the cuneus was statistically similar among populations (Fig. 6; $F_{2, 42} = 2.9$; $P = 0.07$). In Fig. 7, we show photographs of the endocorium obtained from a UG male and a GC male (i.e., males from the two sympatric populations); differences in length and density of setae are easily seen.

mtDNA. Forty-four and 33 of the 45 specimens were successfully sequenced for CO1 and CytB, respectively, yielding 33 combined sequences. These represent five unique haplotypes for CO1 and seven unique haplotypes for CytB and for the combined sequences (Fig. 8). No haplotypes were shared among the three clades (Fig. 8). The sequences of all unique haplotypes can be found in GenBank by accession number (CO1: DQ993256-60; CytB: DQ993261-67). Differences between individuals using the Kimura two-parameter index and number of bp differences as distance metrics are summarized in Table 2 for the combined genes. The maximum difference observed among the seven haplotypes was 32 bp of 766 (4%). Twenty-three polymorphic sites were observed in CO1; seven sites were parsimony informative; all were synonymous substitutions, including 19 in the third codon position and four in the first position (leucine). Of 22 polymorphic sites observed in CytB, 21 were parsimony informative, 17 were third position synonymous, and two were first position synonymous; three first position substitutions corresponded to amino acid replacements.

The NJ trees based on DNA sequence of CO1 (425 bp), CytB (341 bp), and these two genes combined

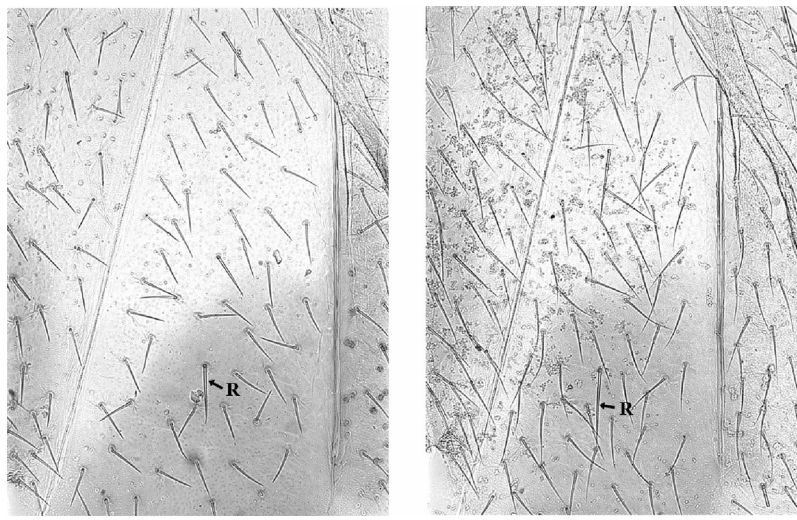

Fig. 7. Photographs of right forewings from the sympatric GC (left) and UG (right) populations showing differences in density and lengths of setae. R, reference seta.

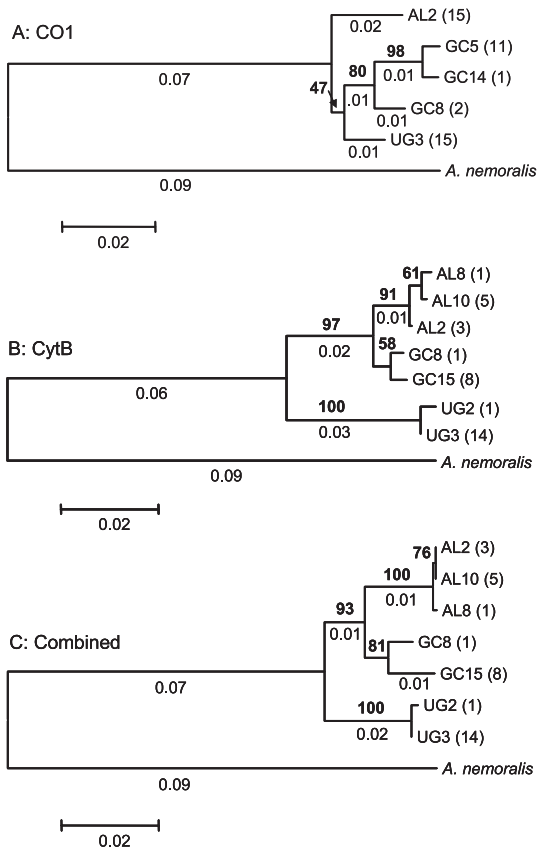

Fig. 8. mtDNA-based NJ trees. All trees were constructed using the Kimura two-parameter distance metric, and those distances are displayed below tree branches. Bootstrap support >50% (5,000 replicates) is displayed above the relevant clades in bold font. Sums of branch lengths are 0.22 for all three trees. AL, GC, and UG, with numbers following those abbreviations depicting our specimen identifiers. Numbers in parentheses adjacent to specimen identifiers indicate the number of specimens (of a maximum 15) observed with identical haplotypes.

(766 bp representing 255 complete codons and 1 bp from incomplete codons) all supported the distinctiveness of the UG, AL, and GC clades as well as the distinctiveness of these clades from the outgroup *A. nemoralis* (Fig. 8). The three trees were virtually identical in length (0.22 distance units, Kimura two-parameter). Bootstrap support (5,000 replicates) for the three clades in NJ trees was ≥47% for CO1 and ≥58% for CytB. Combining the two genes produced seven unique *A. antevolens* haplotypes, and the combined data showed even stronger support for three distinct clades (NJ; branch support 81–100%). Other algorithms (maximum parsimony, maximum likelihood, Bayesian–Markov methods) showed even higher support for the same clades (data not shown). A single maximum parsimony tree from PAUP4 combining the 766 coding characters with the seven *A. antevolens* haplotypes showed identical branch orders as seen in Fig. 8; this tree was characterized by a rescaled consistency index of 0.93, homoplasy index excluding uninformative characters of 0.067, and a retention index of 0.99. We also observed among-specimen variation within the GC clade associated with CO1, and to a lesser extent within the AL clade from differences in CytB (Fig. 8).

Discussion

Previous studies showed that populations of *A. antevolens* diverge in sexual behavior (Horton et al. 2005) and characteristics of the male's genitalia (Horton and Lewis 2005). Even populations occurring in sympatry have been shown to diverge in behavior or morphology. Horton et al. (2005) showed for the AL, GC, and UG populations that insemination success was substantially reduced in interpopulation crosses (0–21% success) compared with success in intrapopulation crosses (64–92%). Indeed, despite vigorous attempts by males to inseminate females, males were 100% unsuccessful at inseminating females in crosses between the two Yakima (GC and UG) populations (Horton et al. 2005). Crosses involving the AL and Yakima bugs showed only limited insemination success, with almost all of the limited success occurring in crosses of GC males with AL females (Horton et al. 2005).

Examination of mensural characters, pubescence, and male genitalia indicates that population divergence noted in the behavioral studies for these study populations (Horton et al. 2005) occurs also in these other phenotypic traits. We have shown in the current study that the two sympatric populations (GC and UG) differed in pubescence (Figs. 6 and 7), tibia

Table 2. Number of nucleotide differences (below diagonal) and Kimura two-parameter distance measure (above diagonal) between seven unique haplotypes seen in the combined data for CytB and CO1

	AL2	AL8	AL10	GC8	GC15	UG2	UG3	*A. nemoralis*
AL2		0.003	0.001	0.021	0.029	0.041	0.039	0.174
AL8	2		0.001	0.024	0.029	0.043	0.042	0.174
AL10	1	1		0.023	0.031	0.042	0.041	0.176
GC8	16	18	17		0.015	0.038	0.036	0.175
GC15	22	22	23	11		0.040	0.039	0.175
UG2	30	32	31	28	30		0.001	0.173
UG3	29	31	30	27	29	1		0.171
A. nemoralis	118	118	119	118	118	117	116	

See Source of Insects and Rearing and Fig. 8 for specimen naming conventions.

length (Fig. 5), cuneus length (Fig. 5), phallus length (Fig. 3), and clasper shape (Fig. 4). Males from the UG population, if compared with males from the GC population, had longer and denser setae on the endocorium, a longer tibia, a longer cuneus, a shorter phallus, and a more robust clasper. Molecular genetic information (Fig. 8) corroborates the behavioral (Horton et al. 2005), morphological, and anatomical data, and it is consistent with the hypothesis that the sympatric GC and UG populations within *A. antevolens* are actually two species. The molecular data are also consistent with the hypothesis that the allopatric AL population is a distinct species.

All insects examined in this study keyed readily to *A. antevolens* in Hill (1957) or Kelton (1978), with bugs from all three sources having the typical *A. antevolens* appearance that separates this species from other North American *Anthocoris* species: hemelytra entirely shiny; antennae not entirely black; and pubescence moderately long and erect (Kelton 1978). Males exhibited the typical phallus of *A. antevolens*, which is distinct in appearance from that seen in all other North American *Anthocoris* spp. other than *Anthocoris musculus* (Say) and *Anthocoris dimorphicus* Kelton & Anderson (T.M.L., unpublished data), despite variation in overall length of the phallus among populations (Fig. 3; Horton and Lewis 2005). Clasper shape is also typical for *A. antevolens* and differs in appearance from all other North American *Anthocoris* spp. except *A. musculus* and *A. dimorphicus* (Kelton 1978; T.M.L., unpublished data).

However, a more careful look at *A. antevolens* here and in previous studies (Horton and Lewis 2005, Horton et al. 2005) has provided convincing evidence that this species is actually composed of an unknown number of species whose external characteristics nonetheless key the insects to *A. antevolens.* The two sympatric Yakima populations in the current study are reproductively incompatible (Horton et al. 2005) and seem to be reproductively isolated from a third Yakima population that has been found almost exclusively on oak in the Yakima valley (Horton and Lewis 2005). Males from the oak population exhibit phallus lengths substantially shorter than reported here for the UG and GC males, even after a generation of rearing in the laboratory on a common diet of pear psylla (compare Fig. 3 with data in Horton and Lewis 2005). It is unclear what evolutionary pressures have produced this three-species complex in the Yakima valley. We have suggested previously (Horton and Lewis 2005) that divergence in phallus length or clasper shape may lead to reproductive incompatibility between populations of *A. antevolens.* There is increasing evidence that sexual selection drives variation in morphology of insect genitalia (Eberhard 1996), potentially leading to reproductive incompatibility and speciation (Via 2001). Whether sexual selection is responsible for variation in morphology of the male's genitalia in *A. antevolens* is not known.

Accurate taxonomic characterization of natural enemies is critical to effective use and understanding of biological control in crop and natural systems (Rosen and DeBach 1973). Elsewhere, we discussed issues concerning biological control in pear orchards that were raised by our findings that *A. antevolens* is actually a complex of species (Horton and Lewis 2005). *A. antevolens* has been listed as a common predator and source of biological control in pear orchards of California (Shimizu 1967), Oregon (Westigard et al. 1968), Washington (Horton and Lewis 2000), and Canada (McMullen and Jong 1967). Results reported here indicate that *A. antevolens* in the fruit-growing regions of central Washington is composed of more than a single species. Efforts are ongoing to assess morphological and genetic variation among populations from a much wider geographic range, to determine whether populations from other regions also comprise a complex of species.

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